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IMPLICATIONS OF TANOAK DECLINE IN FORESTS IMPACTED BY *PHYTOPHTHORA RAMORUM*: GIRDLING DECREASES THE SOIL HYPHAL ABUNDANCE OF ECTOMYCORRHIZAL FUNGI ASSOCIATED WITH *NOTHOLITHOCARPUS DENSIFLORUS*

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ABSTRACT

Invasive plant pathogens are often recognized as serious threats to the maintenance of biodiversity affecting both structure and function of ecosystems. Here, we investigate the potential impact of the invasive pathogen *Phytophthora ramorum* Werres, de Cock & Man in't Veld by using physical girdling of tanoak, *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. H. Oh (Fagaceae), as a surrogate for the disease and to test for changes on the hyphal abundance of ectomycorrhizal fungi. In this study, the flow of phloem to the roots of girdled trees was severed by cutting two narrow incisions (about 10 cm distant) through the inner bark around the circumference of the stem of each tree (fully-girdled), or by cutting two narrow incisions half of the circumference of the tree (half-girdled), to compare with untreated (non-girdled) trees. The hyphal abundance of two common and ecologically important ectomycorrhizal genera (*Cenococcum* and *Tricholoma*) was estimated from the roots and surrounding soil using real-time PCR quantification (TaqMan) assay. A significant decrease in the hyphal abundance from soil was observed in girdled tree plots. In contrast, no similar decrease in the root hyphal abundance was observed. Ectomycorrhizal fungi have a major impact on ecosystem function through their control over decomposition, nutrient acquisition, and mobilization and regulation of succession in plant communities. Given their important function, the decline in EM abundance of tanoak infected by *P. ramorum* will likely disrupt the function and structure of these forests.

Key Words: Ectomycorrhiza, extramatrical mycelium, girdling, *Phytophthora ramorum*, sudden oak death, tanoak.

The dissemination and spread of invasive plant pathogens has increased dramatically over the last century as a result of the inadvertent movement of infected plants (Pimentel et al. 2000; Liebhold et al. 2012). One recent invasion involves the introduction of the microbial pathogen, *Phytophthora ramorum* Werres, de Cock & Man in't Veld, the cause of the disease referred to as Sudden Oak Death or SOD. The pathogen often appears as a lethal canker on tanoak, *Notholithocarpus densiflorus* (Hook. & Arn.) Manos, Cannon & S. H.Oh, and a few oak species: *Quercus agrifolia* Née, *Q. kelloggii* Newb. and *Q. parvula* Greene (all in the Fagaceae), in the central coastal region of California (Garbelotto et al. 2001; Rizzo et al. 2002). Although the origin of *P. ramorum* in forests is unknown, the disease was likely introduced in central California on infected nursery stock and dispersed long distance by the transport of infected plants or locally by wind-blown rain (Davidson et al. 2005; Ivors et al. 2006; Mascheretti et al. 2008; Grünwald et al. 2012). In mixed-evergreen and redwood–tanoak forests of the central coast of California, *P. ramorum* has reached epidemic levels, aided by its rapid sporulation on non-lethal hosts, especially bay laurel, *Umbellularia californica* (Hook. & Arn.) Nutt., during rainy intervals (Davidson et al. 2005). By far, tanoak is the most susceptible host as evidenced by the high recovery of *P. ramorum* from lethal cankers, the thousands of dead tanoak trees observed in coastal forests, and the lack of genetic resistance within tanoak populations (Davidson et al. 2005; Waring and O'Hara 2008; Hayden et al. 2011).

Tanoak, a broadleaf evergreen tree, is a significant component of the low- to mid-canopy strata of mixed-evergreen and coast redwood, *Sequoia sempervirens* Endl., forests in central California and southern Oregon (Waring and O'Hara 2008; Ramage and O'Hara 2010; Ramage et al. 2011). In redwood forests, tanoak is often the dominant

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ectomycorrhizal (EM) host, forming obligate associations with a diverse assemblage of fungi (Kennedy et al. 2003; Bergemann and Garbelotto 2006). This mutualism involves the direct mobilization and translocation of nutrients (especially N and P) from fungi in exchange for carbon derived from plant photosynthesis (see review by Leake et al. 2004). In a survey of mature (ca. 60 yr) tanoak roots in mixed Douglas-fir–tanoak forests in northern California, over 250 species of root-associated fungi were estimated from a 2-km^2 area (Bergemann and Garbelotto 2006). The vast majority of root-associated fungi shared affinity with well-known EM fungi of the Ascomycota and Basidiomycota (dominant fungi include *Cenococcum geophilum* Fr., and species of *Russula, Lactarius,* and *Tomentella*) (Bergemann and Garbelotto 2006).

EM fungi are of major importance in microbial communities because of their role in plant-nutrient acquisition and regulation of succession in plant communities (Nara and Hogetsu 2004; Nara 2006). Given their role, the question arises as to what will be the impacts of infection by *P. ramorum* on tanoak EM composition and function? The decline of EM function is likely to be more pronounced in forests where tanoak is the dominant EM host (e.g., tanoak–redwood forests) because of the reduced EM inoculum. In contrast, other EM hosts may counter the effects of declining abundance if they have similar EM assemblages. In tanoak–Douglas-fir forests, the majority of EM species found on the roots of tanoak seedlings were also found on Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco (Kennedy et al. 2003). In contrast, coast redwood forms associations with arbuscular mycorrhizal (AM) fungi, including species in the Glomeromycota (Wang and Qiu 2006), and ericaceous shrubs form assemblages with a different assemblage of fungi. Although AM, EM, and ericoid mycorrhizae are able to mobilize and transfer N and P, the enzymatic capabilities of EM allow for greater N acquisition by hydrolysis of organic polymers in litter and soil (Read and Perez-Moreno 2003; Talbot and Treseder 2010).

The objectives of this study are to simulate the mortality caused by *P. ramorum* through physical girdling of trees and assess the EM biomass from roots and soil. Because of the dependence of carbon assimilate from the tanoak trees to fuel the growth and activity of EM fungi, it would be expected that girdling should decrease the EM biomass of roots and soil in plots with girdled trees. Here, we test this hypothesis using a quantitative PCR (qPCR) assay to target hyphae of EM fungi from tanoak roots and the surrounding soil. Due to the complexity of the EM assemblage in the Douglas-fir–tanoak forest (Kennedy et al. 2003; Bergemann and Garbelotto 2006), we focus on sampling two EM genera (*Cenococcum* and *Tricholoma*) that vary with respect to reproduction (asexual or sexual), extramatrical hyphal abundance, and their prevalence in the tanoak root community. *Cenococcum geophilum* is the most frequently encountered taxon in tanoak (Kennedy et al. 2003; Bergemann and Garbelotto 2006). The mycelia of *C. geophilum* are considered a short-range exploration type due to the short hyphal extensions observed on EM roots (Agerer 2001). In contrast, *Tricholoma* species maintain extensive aggregates of mycelium that may be connected to medium-distance transport structures called cords (Agerer 2001). Here, we compare the abundance of the two genera from both substrates after disruption of the carbon flow from leaves to roots by girdling trees.

Materials and Methods

Study Site, Experimental Design, and Sampling

The study was conducted on privately owned Douglas-fir–tanoak forest near the town of Whitethorn, California (Humboldt Co.), USA (40°00′30″N, 123°57′00″W) (Fig. 1A). The selection of non-girdled and girdled (half-girdled and fully-girdled) followed a partially randomized block design. Five blocks (1600 m^2) were chosen on the basis of similar densities of tanoak and the absence of Douglas-fir and ericoid trees or shrubs (*Gaultheria shallon* Pursh, *Vaccinium ovatum* Pursh (Fig. 1B). Baseline sampling was performed to examine EM composition prior to girdling in January 2003 (Bergemann and Garbelotto 2006). Next, non-girdled tree plots were assigned to the center of each of the five blocks in an attempt to balance the intrusion of living roots into treated plots (Fig. 1C). The remaining two treatments (half-girdled and fully-girdled) were randomly assigned to the two plots within each block (Fig. 1C). The phloem of girdled trees was severed with a chainsaw by cutting two narrow incisions (about 2 cm in depth and 10 cm distant) around the circumference of the stem of each tree (fully-girdled) or by cutting two narrow incisions half of the circumference of the tree (half-girdled) for comparison with non-girdled trees. Half-girdling (girdling half the circumference of the tree) was performed to determine whether the reduction of carbon assimilate exhibited properties of similar severity under a partial flux in the phloem. Root tips and soil were sampled from each plot, five (June 2003), nine (October 2003), and 13 (February 2004) months after girdling. Two, 6-m, perpendicular transects were placed by random selection of two intersecting points along 15 m × 15 m grids positioned in the center of each plot (Fig. 1D). Three soil cores were sampled at 2-m intervals (n = 6) along each transect from each plot (n = 15) (Fig. 1D), using PVC pipe 20 cm in length with an internal

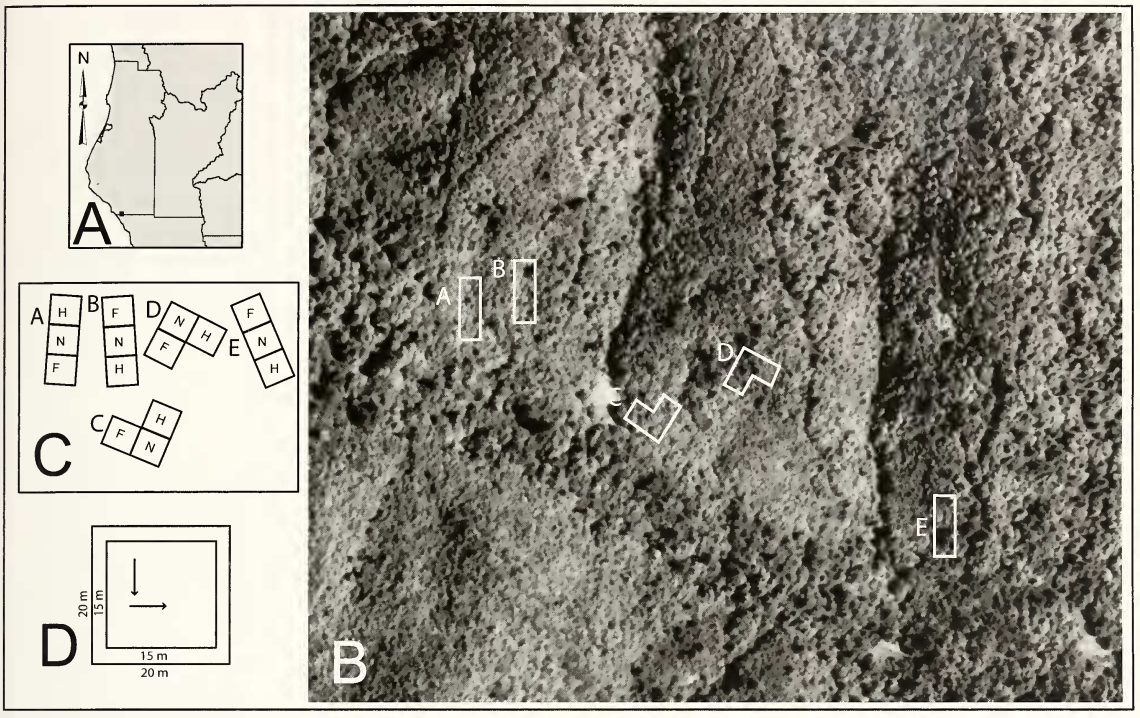

FIG. 1. Location of tanoak study sites and experimental block layout. A. Location of the study site in southern Humboldt County, California. B. Aerial photograph showing the location of blocks. C. Experimental layout of each block after simple randomization of treatments. Control plots were positioned in the center of each block and experimental plots (N = 'non-girdled', F = 'fully-girdled', H = 'half-girdled') were randomly assigned. D. Layout of plot design showing the grid method used for establishing two, 6 m transects for collecting soil and roots positioned towards the center of the plot.

diameter of 2.5 cm and transported on ice to the lab at UC Berkeley within 48 hr. Each core was divided into 10 sections of equal volume (each 16 cm^3), and two subsections were randomly selected from the 10 subsections using simple random number generation and excised using an electric saw. Soil cores were stored at 4°C until processed. Soil and roots were lyophilized for approximately 36 hr, after which all fine root mass (<2 mm in diameter) was separated from soils using a forceps, then weighed and stored at −80°C.

Quantification of Ectomycorrhizal Abundance Using TaqMan Assays

The gene amplified from *Cenococcum* and *Tricholoma* in TaqMan assays was the nuclear ribosomal large subunit DNA (28S nrDNA), and the conserved regions for primer design were based on alignments of EM sequences generated for phylogenetic analysis of tanoak EM communities (Bergemann and Garbelotto 2006). Primers and probes were designed using Primer 3 ver. 0.6 (Rosen and Skaletsky 2000) with the following parameters: 1) product size 50–90 bp; 2) primer size = 18–27 bp; 3) primer T_m = 58–60°C; 4) Max primer T_m difference = 2; 5) hybrid oligo size = 21–30 bp; 6) hybrid oligo T_m = 67–70°C (Table 1). Primers and dual labeled probes with a 5′ fluorophore (6FAM) and 3′ black hole quencher (BHQ) were synthesized by Operon (Huntsville, AL) (Table 1).

Genomic DNAs were extracted from pooled root sections and 10% of the soil mass was subsampled using the UltraClean Soil DNA Kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's recommendations. DNA quantity and detection was measured using the 5′ fluorogenic exonuclease (TaqMan) qPCR assay that combines the use of an internal probe with standard PCR primers (Heid et al. 1996). The probe is labeled with a 5′ fluorescent reporter dye and a 3′ quencher molecule. Once the reporter is bound and the *Taq* enzyme elongates the target sequence, the reporter dye is released and fluorescence is detected (Heid et al. 1996). Two standards, for *Cenococcum* (N72) and *Tricholoma* (S1) shown in Table 1, were generated for standard quantification by 10-fold serial dilution (10^{-1}–10^{-5} pg) after purification of plasmid DNA (Qiagen Plasmid Mini-Prep, Valencia, CA). The quantity of DNA was estimated using a UV spectrophotometer (Bio-Rad, Hercules, CA). To

TABLE 1. SEQUENCE IDENTIFIERS, GENBANK ACCESSIONS, TAQMAN PRIMERS, AND PROBES AND CHARACTERISTICS OF TWO ECTOMYCORRHIZAL GENERA (*CENOCOCCUM* AND *TRICHOLOMA*) QUANTIFIED IN GIRDLING EXPERIMENTS. References: 1) Massicotte and Trappe 1992; 2) Shinohara et al. 1999; 3) Gill et al. 2000; 4) Agerer 2001; 5) Jany et al. 2002; 6) Huai et al. 2003; 7) Chapela and Garbelotto 2004; 8) Douhan and Rizzo 2005; 9) Gryta et al. 2006; 10) Carriconde et al. 2008b; 11) Carriconde et al. 2008a; 12) Jargeat et al. 2010; 13) Grubisha et al. 2012.

Genus	Species composition	Sequence identifier and GenBank accession numbers	Primer and probe sequences (5′–3′)	Genetics, morphology, reproduction
Cenococcum	22 ITS sequence types	P62 - DQ273447	Cen F: CCAGCATC-CTAGCCGAAG	*Reproduction*–no known sexual stage, form sclerotia (1)
		A27 - DQ273448	Cen Hyb: GGACCTCA-GTTCAGGCTGGCC	*Morphology of EM*–smooth, thick mantles with short-emanating hyphae, no rhizomorphs (4)
		F39 - DQ273449	Cen R: GTTATAGC-CCGTTGCGAAAT	*Dispersal of clerotia*–water, birds, insects, human-mediated transport of plants, rodents (2)
		A4 - DQ273450		*Distribution*–widespread, all continents (2)
		N78 - DQ273451 N72 - DQ473308		*Genetic diversity*–genotypic diversity high in populations studied (5, 8); some form cryptic species (8)
Tricholoma	*T. magnivalere*	G58 - DQ273556	Trich F: CGGACTACC-AGGGCTTATG	*Reproduction*—sexual, epigeous sporocarps (6)
	T. saponaceum	R24 - DQ273557	Trich Hyb: GCGCTCTCAA-AGAGTCGAGTTGTTTG	*Morphology of EM*–undifferentiated to smooth mantle, some with cords (3); *T. magnivalere* and close allies form dense hyphal mats (shiros) (7); well-developed extramatrical mycelia observed in several species (4)
	T. portentosum	F16 - DQ273558	Trich R: CCCATTTAGAG-CTGCATTCC	*Dispersal*–spores in epigeous fruit bodies (7)
	Tricholoma sp. 1	S1 - DQ273559		*Distribution*–widespread, continental (1, 7)
	Tricholoma sp. 2	W74 - DQ273560		*Genetic diversity*–many genets in some species (2, 6, 10); few genets in some species (9); some form cryptic species (11, 12, 13)

Table 2. Optimized Parameters for Quantitative TaqMan Assays of *Cenococcum* and *Tricholoma*.

Genus	T_a 56–62°C	Primer concentration	Probe concentration/ label	Root template	Soil template
Cenococcum	58°C	260 nM	260 nM/6FAM	1:1000	1:5000
Tricholoma	57°C	200 nM	260 nM/6FAM	1:100	1:1000

test the specificity of the TaqMan primers and probes, all plasmids with target 28S sequences from each genus were diluted (1:5000) and PCR amplified (Table 1). Next, we assessed whether the primers and probes would amplify non-target EM fungi after dilution within the quantified range of standards (5×10^{-5}) (Appendix 1). All TaqMan reactions were performed in a 15 µl volume containing 1× TaqMan Universal Master Mix (ABI, Foster City, CA), 200–260 nm of forward and reverse primers, 260 nM dual-labeled probes and 5 µl of template DNA diluted 1:100 to 1:5000 (Table 2). In the absence of cross reactivity, DNA template quantities were estimated using the MyiQ (6FAM) Real-Time PCR Detection System (Hercules, CA) with a single cycling parameter. Cycling conditions were as follows: 1 cycle at 50°C for 10 min, 1 cycle at 95°C for 1 min 30 sec, 55 cycles at 95°C for 15 sec and T_a for 1 min (Table 2), followed by a hold at 4°C for 30 sec. Standard DNA was quantified within the range of 5×10^{-1}–5×10^{-5} pg for both *Cenococcum* and *Tricholoma*. Root and soil DNA templates were optimized for template quantity (i.e., diluted within the quantified range of the standards), and PCR efficiency (Table 2). To account for error in enzyme kinetics between the roots and soil, an environmental negative (either root or soil DNA extract with no target template) was added to the standards using the same dilution factor of the PCRs (Table 2). Threshold (C_t) values (the level at which template fluorescence exceeds background fluorescence) were calculated for each TaqMan assay using BioRad MyiQ Real-Time Detection System software using the maximum correlation coefficient approach (Hercules, CA). With this approach, the threshold is automatically determined to obtain the highest possible correlation coefficient for the standard curve under environmental PCR conditions. The standard curve was used to estimate the sample quantity of DNA from roots and soil DNA using the same software described above. If samples displayed irregular curvatures during the amplification cycles, they were removed prior to calculation of starting quantity.

The qPCR approach was to amplify fungal-specific hyphal abundance of *Cenococcum* and *Tricholoma* with separate reactions with separate primers from genomic DNA and to calculate hyphal biomass per sample by quantifying the concentration of starting DNA (pg) divided by the dry weight (g) of root and soil and multiplied by an appropriate dilution factor. After pooling biomass of both sections from a single core (by summation of the biomass quantities from each section), a repeated measures ANOVA (using three-way RM-GLM with time sampled as within factor) was used to analyze the effects of the girdling in time on log (Y+1) transformed values of EM abundance of each taxon (*Cenococcum* and *Tricholoma*) from root and soil. If Mauchly's criterion indicated rejection of the compound symmetry assumption (i.e., the lack of independence between the subject factor and the treatment), probability values were estimated after adjusting the number of the degrees of freedom for the F distribution using the Greenhouse–Geiser correction. Analyses were conducted using SPSS 17 (Chicago, IL) and considered significant at $P < 0.05$.

Results

The repeated-measures analysis showed a significant reduction of EM soil hyphal biomass in girdled treatments (fully-girdled, half-girdled) compared to the control (non-girdled) (Table 3, Fig. 2). In contrast, we observed no significant reduction of EM root hyphal biomass in girdled plots (Table 3, Fig. 2). Additionally, EM soil hyphal biomass varied among sampling periods with greater soil hyphal biomass in early summer (June) compared to both the early fall (October) and late winter (February) sampling periods (Table 3, Fig. 2). EM root hyphal biomass also varied across seasons but a significant interaction between taxon and time indicates that seasonal variation is dependent on the taxa (Table 3, Fig. 3).

Discussion

The main objective of this study was to determine whether reducing carbon flow from the leaves to the roots by girdling would cause a decline in the EM hyphal abundance. Our study demonstrates that the extramatrical hyphae of tanoak roots decrease in abundance after carbon depletion by girdling, whereas the root hyphal biomass is unaffected over the same duration of sampling. Our results are consistent with previous experiments that showed that a reduction of carbon assimilate from the leaves to the root by girdling resulted in a significant decline in the activity and biomass of soil microbes, mainly due to the loss of EM fungi (Högberg et al. 2001; Högberg and Högberg 2002; Göttlicher et al.

TABLE 3. RESULTS OF THE REPEATED MEASURES ANOVA. T = time of sample (five, nine and 13 months after girdling. F values and significance levels from the repeated measures ANOVA are shown for all main effects and their interactions: *0.01 < P < 0.05, **0.001 < P < 0.01, ***P < 0.001, ns = non significant. When Mauchly's P < 0.05, Greenhouse–Geisser estimates were used to correct for sphericity.

		Repeated measures GLM							
		T		T × Taxon		T × Treatment		T × Taxon × Treatment	
	Mauchly's P	F	P	F	P	F	P	F	P
root hyphal abundance (pg DNA g^{-1} root)	0.015	16.706	***	5.532	**	0.987	ns	0.912	ns
Taxon		41.417	***						
Treatment		0.792	ns						
Taxon × Treatment		0.594	ns						
soil hyphal abundance (pg DNA g^{-1} soil)	0.019	5.749	**	2.165	ns	0.124	ns	0.546	ns
Taxon		118.680	***						
Treatment		3.736	*						
Taxon × Treatment		0.932	ns						

2006; Scott-Denton et al. 2006; Weintraub et al. 2007; Feng et al. 2009; Kaiser et al. 2010). This study lends further support that girdling severely limits the growth and maintenance of the extramatrical hyphae, the main conduit for decomposition and nutrient acquisition and mobilization (Leake et al. 2004).

Microcosm and field studies of EM fungi have shown that up to 20–30% of net photosynthate is transferred to the fungal associate to support their growth and activity of the extramatrical mycelium (Söderström 1992; Högberg and Högberg 2002; Hobbie 2006). Furthermore, the extramatrical mycelia account for up to 80% of the total EM biomass and nearly 30% of the microbial biomass in temperate forests (Wallander et al. 2001; Högberg and Högberg 2002). Such an investment in the maintenance of these extensive mycelial networks is required for nutrient acquisition and mobilization (Leake et al. 2004). Here, the disruption of a carbon supply to the roots by girdling reduced the soil EM extramatrical mycelium.

Many EM species possess a broad class of the enzymes for acquisition and uptake of N and P from organic polymers (Read and Perez-Moreno 2003; Leake et al. 2004; Courty et al. 2010; Talbot and Treseder 2010). With the loss of a direct source of carbon assimilate from the host and after girdling, the enzymatic activity of proteases and cellulases show similar decreases (Kaiser et al. 2010). Cellulases are a suite of enzymes that enhance degradation of cellulose allowing entry of hyphae into plant litter (a feature typical of saprotrophic fungi). In general, EM fungi possess a reduced set of genes encoding cellulose-degrading enzymes (Martin et al. 2008; Nagendran et al. 2009; Wolfe et al. 2012) that contribute to poor cellulose degradation compared to that of saprotrophic fungi (Baldrian 2009). Rather, the decrease in the activities of cellulases is likely due to the elimination of rhizosphere carbon inputs from the roots of girdled trees (Subke et al. 2004; Kaiser et al. 2010). In contrast, decreases in the abundant EM extracellular proteinases that hydrolyze proteins found in organic matter after girdling are a likely consequence of a decline in the abundance of EM soil extramatrical mycelia (Read and Perez-Moreno 2003; Talbot and Treseder 2010).

Although soil EM hyphal abundance was lower in girdled trees, we observed no similar decrease in EM hyphal root abundance after 13 months. Roots are known to be essential for the storage of starch used by EM and plants in the absence of a supply of carbon assimilate from the leaves (Pena et al. 2010). Such reserves are seasonally dependent; the depletion of starch reserves that occurs during spring bud break and the repletion during winter months of plant dormancy are common occurrences (Regier et al. 2010). In girdled trees, starch reserves are typically depleted over time (Bhupinderpal-Singh et al. 2003; Pena et al. 2010; Regier et al. 2010). For example, Pena et al. (2010) measured starch concentrations in the fine roots of girdled *Fagus sylvatica* L. trees and found quantities to be about one-quarter of that estimated from the roots of non-girdled trees. After two years, the roots of girdled *P. sylvestris* trees, soluble carbohydrates were reduced to a small fraction of that found in non-girdled trees (Bhupinderpal-Singh et al. 2003).

We found no significant differences in EM abundance between girdled (half-girdled and fully-girdled) and non-girdled trees. One possible explanation for this result is that the transport of carbon from the leaves to roots is maintained in fully-girdled trees near levels of half-girdled trees. In *F. sylvatica*, a low supply of carbon assimilate bypasses the girdle through transport in axial parenchyma cells, yet these fractions are insignif-

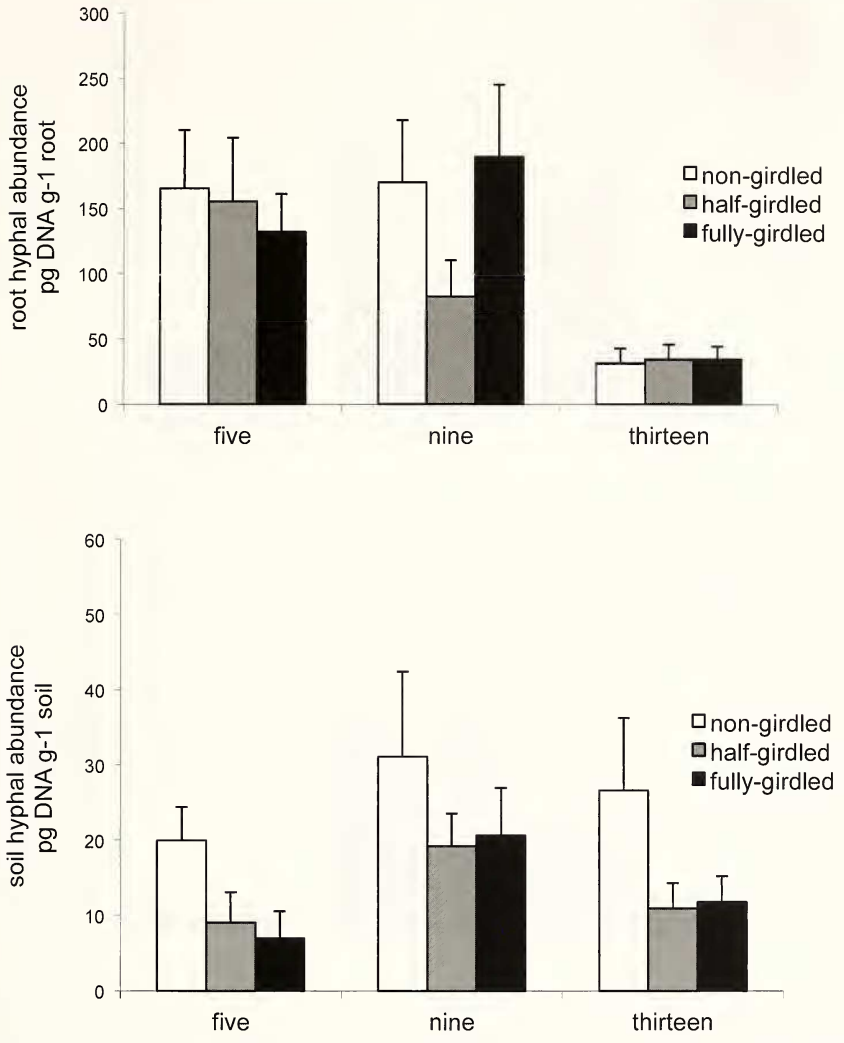

FIG. 2. Ectomycorrhizal hyphal abundance from roots and soil five (June 2003), nine (October 2003) and thirteen (February 2004) months after girding *Notholithocarpus densiflorus* trees. Mean hyphal abundance for each treatment (+1 SE) are graphed. Girdling decreased the ectomycorrhizal soil hyphal abundance did not similarly affect root hyphal abundance.

icant for supporting the EM mycelia of roots (Druebert et al. 2009). Therefore, a more plausible explanation for the similar decline in EM soil hyphae in girdled trees is that carbon resources after girdling are directed towards secondary metabolic pathways (e.g., production of phenolic compounds) in response to wounding (Maunoury-Danger et al. 2010). This could explain why wounded (half-girdled and fully-girdled) trees exhibit similar decreases in EM abundance.

Another interesting observation from this study was that *Cenococcum* was found to be the root dominant in terms of frequency (Bergemann and Garbelotto 2006), and, in this study, was the mycelial dominant from roots and soil. Overall dominance by *Cenococcum* on roots is likely a reflection of its competitive ability for rapid colonization either by numerous sclerotia (Dahlberg et al. 1997) or proliferation via root-to-root contacts. We expected that *Cenococcum* would occupy a high proportion of EM root biomass due to the high frequency observed on tanoak roots (Bergemann and Garbelotto 2006). In this study, we found evidence that the root hyphal abundance of *Cenococcum* varied across seasons and was unaffected by girdling, a result consistent with the girdling experiments of *F. sylvatica* that showed no effect on EM frequency after girdling (Danneman et al. 2009). However, the greater soil hyphal abundance of *Cenococcum* compared to *Tricholoma* was unexpected given their short-emanating hyphae (Agerer 2001). The higher density of root occupation for *Cenococcum* is a

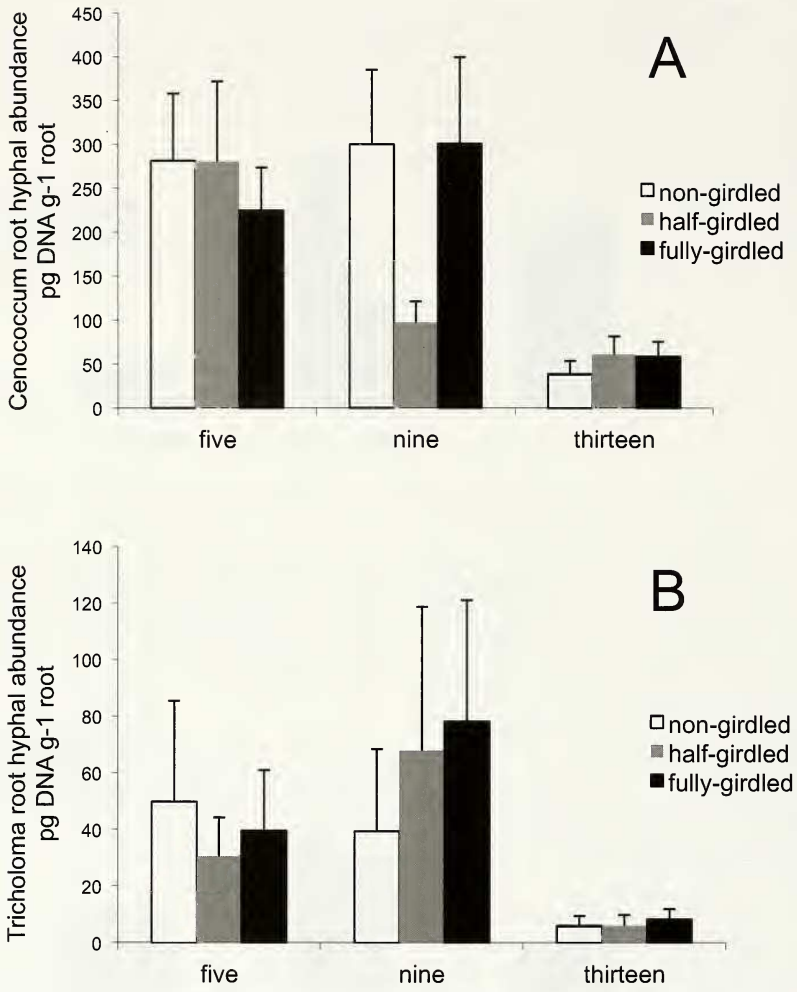

FIG. 3. Root and soil hyphal abundance of *Cenococcum* and *Tricholoma* five (June 2003), nine (October 2003) and thirteen (February 2004) months after girding *Notholithocarpus densiflorus* trees. Means (+1 SE) are graphed. A. *Cenococcum* root hyphal abundance. B. *Tricholoma* root hyphal abundance. C. *Cenococcum* soil hyphal soil abundance. D. *Tricholoma* soil hyphal abundance. A significant taxon × time interaction was observed for ectomycorrhizal root hyphal abundance but not for soil hyphal abundance.

likely explanation for its observed dominance in soil.

Several factors should be taken into consideration when comparing the biomass of taxa and between substrates using qPCR assays. For one, the number of rDNA copies varies considerably between EM species, and the DNA starting quantity inferred from these assays is a function of both the number of copies detected and the template quantity (Landeweert et al. 2003; Raidl et al. 2005). In addition, only a few qPCR studies have found a significant relationship between hyphal abundance and fungal biomass inferred from qPCR (Raidl et al. 2005). Also, a potential error arises from the variation in biomass estimates in different substrates (roots and soil) because of the variation in qPCR kinetics by the various environmental inhibitors in either substrate. Our approach was designed to minimize the inherent variation between substrates by simulating environmental PCR conditions; however, lower hyphal biomass estimates in soil compared to roots may be due to greater PCR inhibition in soil substrates. Despite these limitations, this study demonstrates that this assay affords sensitive quantification of EM genera from mixed-species substrates for comparisons across time and after girdling.

With the observed decline in abundance of the EM extramatrical mycelia, we can only speculate about the possible disruptions to EM function and ecosystem productivity. For example, we expect that plant growth and establishment may be altered with a reduction of EM inoculum (Perry et al. 1989; Nara and Hogetsu 2004), and a decline in EM species richness may accompany

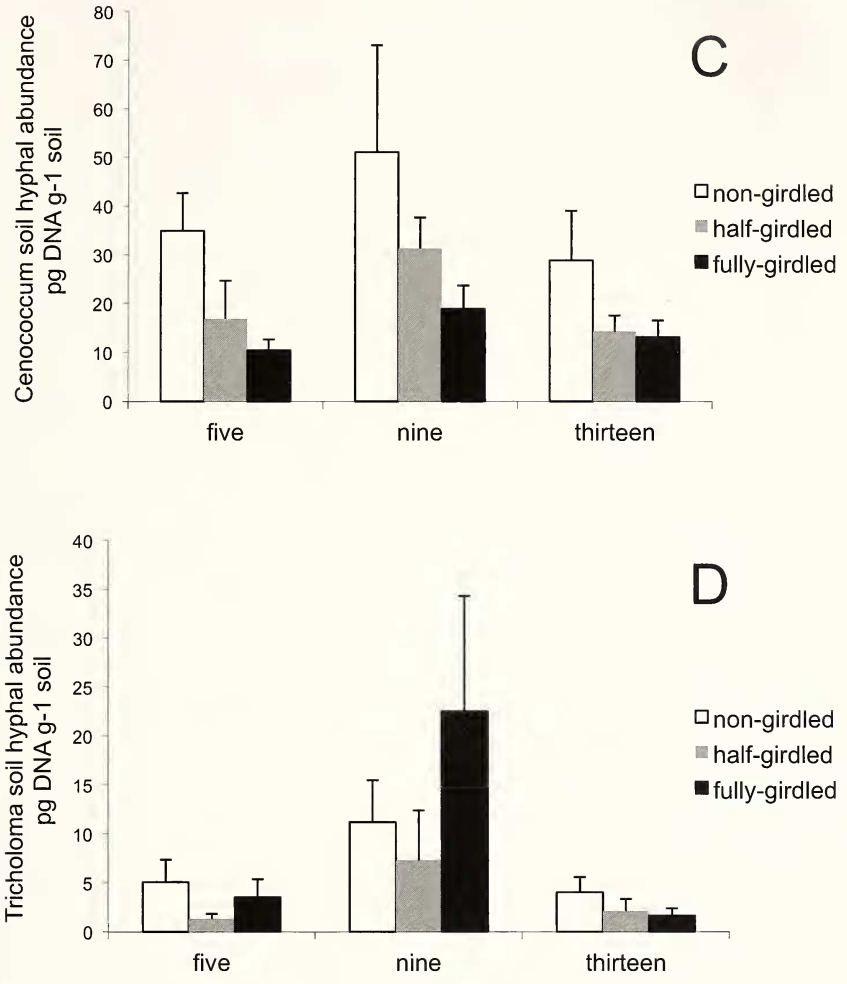

FIG. 3. Continued.

the loss of carbon supply to the roots by girdling (Kaiser et al. 2010; Pena et al. 2010). Also, variation in host-nutrient concentrations after inoculation with EM fungi provides evidence that nutrient acquisition and mobilization is not equivalent among species (Nara 2006), and a decline in carbon assimilate by girdling reduces the activity of EM proteinases that assimilate nutrients from organic matter (Chalot and Brun 1998; Read and Perez-Moreno 2003; Talbot and Treseder 2010). Whether intact mycorrhizal mutualisms in other EM plant species (e.g., Douglas-fir and ericoid plants) will offset the decline in EM richness and function in soils is also unexplored in the present study. From this research, we have gathered evidence that shows a decrease in the extramatrical biomass of EM fungi after girdling; the potential for disruption of ecosystem productivity in stands infected by *P. ramorum* should be considered in future research.

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Appendix 1. Taxon, clone identifiers, and GenBank accessions of nuclear ribosomal large subunit sequences from *Notholithocarpus densiflorus* roots to test the specificity of target template DNA and cross-reactivity of non-target template DNA in TaqMan assays.

Fungal taxon	Clone source - GenBank accession	Fungal taxon	Clone source - GenBank accession	Fungal taxon	Clone source - GenBank accession
Alpova	G5 - DQ273499	*Cortinarius*	Y1 - DQ273507	*Piloderma*	N3 - DQ273488
Amanita	O16 - DQ273481	*Cortinarius*	N43 - DQ273508	*Ramaria*	O4 - DQ273519
Amanita	K11 - DQ273482	*Cortinarius*	O25 - DQ273509	*Russula*	Q52 - DQ273528
Amphinema	C13 - DQ273487	*Cortinarius*	S4 - DQ273510	*Russula*	K10 - DQ273529
Ascomycota	F22 - DQ273445	*Entoloma*	D16 - DQ273516	*Russula*	P50 - DQ273530
Ascomycota	F6 - DQ273446	*Galiella*	S7 - DQ273473	*Russula*	Q1 - DQ273531
Basidiomycota	G20 - DQ273490	Gomphaceae	G3 - DQ273518	*Russula*	N17 - DQ273534
Basidiomycota	D34 - DQ273491	Gomphaceae	C8 - DQ273520	*Sebacina*	X9 - DQ273535
Basidiomycota	C11 - DQ273492	Gomphaceae	D43 - DQ273521	*Sebacina*	Y2 - DQ273536
Boletopsis	X33 - DQ273552	*Hydnellum*	D1 - DQ273539	*Sebacina*	O17 - DQ273537
Boletus	A34 - DQ273498	*Hygrophorus*	A61 - DQ273522	*Sebacina*	N11 - DQ273538
Boletus	F12 - DQ273495	*Inocybe*	C25 - DQ273513	Sordariomycetes	D11 - DQ273475
Byssocorticium	A1 - DQ273484	*Inocybe*	A6 - DQ273514	Sordariomycetes	D38 - DQ273477
Byssocorticium	X34 - DQ273485	*Inocybe*	F59 - DQ273515	Thelephorales	F45 - DQ273548
Cadophora	F13 - DQ273453	*Lachnum*	D12 - DQ273458	*Tomentella*	A73 - DQ273540
Cadophora	C34 - DQ273455	*Lachnum*	F3 - DQ273459	*Tomentella*	A20 - DQ273541
Cantharellales	P4 - DQ273504	*Lactarius*	A16 - DQ273524	Thelephorales	A14 - DQ273542
Capronia	C14 - DQ273472	*Lactarius*	A22 - DQ273525	*Tomentella*	C70 - DQ273543
Cenococcum	N72 - DQ473308	*Macowanites*	B11 - DQ273399	*Tomentella*	A21 - DQ273544
Cenococcum	A27 - DQ273448	*Melanogaster*	B8 - DQ273497	*Tomentella*	W54 - DQ273546
Cenococcum	A4 - DQ273450	*Mortierella*	F58 - none	Thelephorales	D3 - DQ273547
Cenococcum	N72 - DQ473308	*Neonectria*	D35 - DQ273479	Thelephorales	G1 - DQ273549
Cenococcum	A31 - N/A	Pezizomycotina	A8 - DQ273465	*Tomentella*	A17 - DQ273551
Cenococcum	B6 - N/A	Pezizomycotina	G60 - DQ273462	*Tomentella*	F57 - DQ273553
Cenococcum	B19 - N/A	Pezizomycotina	P2 - DQ273464	*Tricholoma*	G58 - DQ273556
Cenococcum	F20 - N/A	Pezizomycotina	N8 - DQ273466	*Tricholoma*	R24 - DQ273557
Cenococcum	G14 - N/A	Pezizomycotina	X35 - DQ273467	*Tricholoma*	F16 - DQ273558
Cenococcum	N69 - N/A	*Phialocephela*	F11 - DQ273456	*Tricholoma*	S1 - DQ273559
Cenococcum	T33 - N/A	*Phialophora*	D44 - DQ273470	*Tricholoma*	W74 - DQ273560